

# A method for improved glycerol utilization in yeast

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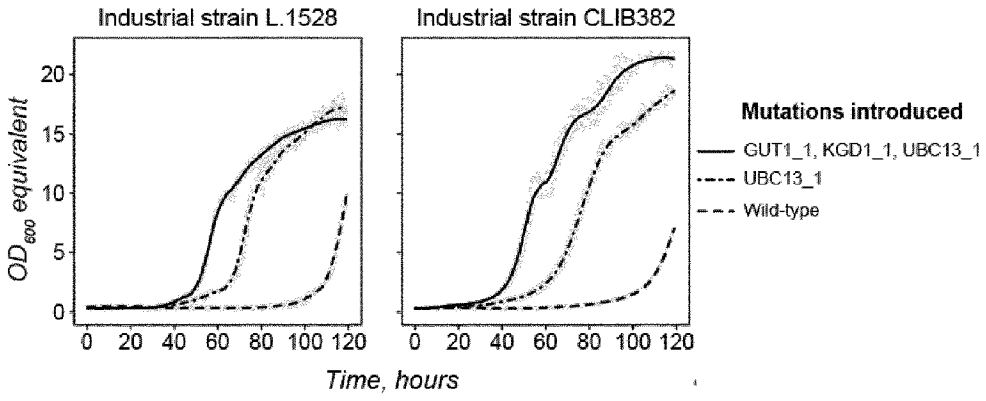
Figure 3




(57) Abstract: The invention provides GM yeast strains for use as cell factories that are capable of using the waste product, glycerol, as sole carbon source, without the addition of an amino acid supplement. The invention additionally teaches how to modify existing yeast strains to have these advantageous properties, whereby the level of glycerol kinase activity (EC:2.7.1.30) is increased and the expression of genes encoding functional alpha-ketoglutarase complex activity (EC:2.7.1.30) and E2 ubiquitin-conjugating enzyme activity (EC:2.3.2.23) are inactivated.

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**TITLE: A method for improved glycerol utilization in yeast**

**Field of the invention**

The invention provides GM yeast strains for use as cell factories that are capable of using the waste product, glycerol, as sole carbon source, without the addition of an amino acid supplement. The invention additionally teaches how to modify existing yeast strains to have these advantageous properties.

**Background of the Invention**

Micro-organisms, in particular yeast and fungi, are widely used for the production of valuable compounds, including biofuels, small chemicals, pharmaceuticals and nutraceuticals. This has been facilitated by advances in yeast and fungal genetic engineering, with the successful introduction of genes encoding diverse heterologous single and multi-step metabolic pathways into the cells of the yeast or fungal host, allowing for an increasing diversity of compounds to be produced. The use of yeast or fungi, for example *Saccharomyces*, as a cell factory has raised awareness of the need to reduce production costs, in particular the ongoing costs of nutrients for cell cultivation. Yeast strains capable of growth on cheap carbon sources, without the need to supply complex supplements such as a source of amino-nitrogen, would reduce production costs. Bio-waste products from manufacturing processes can provide an attractive cheap source of carbon. In particular, glycerol is an important bio-waste product, since it is a by-product of bio-ethanol, oleo-chemical and bio-diesel production.

Although yeasts such as *Saccharomyces cerevisiae* are capable of degrading glycerol by purely oxidative metabolism, when placed in a medium also comprising sugar, the cells are incapable of consuming the glycerol until the sugar has been consumed, due to catabolic repression. Furthermore, when cultured under oxidative conditions on synthetic media with glycerol as sole carbon source, the cells consume the glycerol extremely slowly, which is not compatible with their use as a cell factory on an industrial scale.

Glycerol utilization, in for example *S. cerevisiae*, is facilitated by the uptake of extracellular glycerol via a glycerol/$H^+$-symporter encoded by STL1. Once inside the cell, glycerol is phosphorylated by a glycerol kinase encoded by

GUT1 resulting in the formation of L-glycerol-3-phosphate. A $FAD^+$-dependent glycerol-3-phosphate dehydrogenase, encoded by GUT2 and located at the outer surface of the inner mitochondrial membrane, then oxidises L-glycerol-3-phosphate to dihydroxyacetone phosphate (DHAP) and the electrons are then directly transferred via $FADH_2$ to the electron transport chain. DHAP then enters directly into the glycolytic pathway via glyceraldehyde-3-phosphate. The three glycerol catabolic pathway genes (STL1, GUT1 and GUT2) are reported to be genetic determinants of a glycerol-growth phenotype in yeast (5). Additionally, specific alleles at the UBR2 and SSK1 loci are additionally reported to contribute to a glycerol-growth phenotype in yeast (5). Introduction of the identified alleles of the genetic determinants (GUT1, UBR2 and SSK1) into a laboratory yeast strain (CEN.PK113-1A), by reverse engineering, generated a strain having a glycerol-growth phenotype. However, the maximum reported growth rate of the strain on a mineral medium with glycerol was 0.08 $h^{-1}$ (5).

By comparison, a maximum specific growth rate of 0.42 $h^{-1}$, has been reported for industrial baker's yeast strain DS28911 grown on a defined mineral medium with vitamins and glucose as carbon source (6).

Accordingly, there remains a real need for novel yeast strains having a glycerol-growth phenotype that has an improved capacity to use glycerol as sole carbon source, reflected in a shorter lag period, a higher growth rate, and greater biomass yield.

**Summary of the invention**

The present invention provides a genetically modified yeast with improved glycerol catabolism, wherein the yeast comprises:

a transgene gene encoding a polypeptide having glycerol kinase activity (EC:2.7.1.30), preferably where the amino acid sequence of the polypeptide has at least 80% amino acid sequence identity to SEQ ID No.: 2, or

a mutant endogenous gene encoding a mutant polypeptide having enhanced glycerol kinase activity (EC:2.7.1.30) as compared to a parent endogenous gene from which the mutant gene was derived, wherein the amino acid sequence of said mutant polypeptide has at least 80% amino acid sequence identity to SEQ ID No.: 4, and wherein amino acid residue 572 is Q (where

the parent gene encodes an amino acid sequence having at least 80% amino acid sequence identity to SEQ ID No.:2 wherein amino acid residue 572 is E);

and wherein one or more genes in said yeast required for expressing a functional alpha ketoglutarase complex are inactivated, wherein said one or more genes is selected from the group consisting of:

a gene encoding a polypeptide having alpha-ketoglutarate a gene encoding dehydrogenase enzyme activity (EC: 1.2.4.2);

a gene encoding a polypeptide having dihydrolipoyl transsuccinylase (EC 2.3.1.61); and

a gene encoding a polypeptide having dihydrolipoamide dehydrogenase (EC:1.8.1.4)

and

wherein a gene in said yeast encoding a polypeptide having E2 ubiquitin-conjugating enzyme activity (EC:2.3.2.23) is inactivated.

The invention further provides a method for enhancing glycerol metabolism in a yeast strain comprising:

a. inserting a transgene gene encoding a polypeptide having glycerol kinase activity (EC:2.7.1.30) or,

- modifying or substituting an endogenous GUT1 gene to encode a mutant polypeptide having enhanced glycerol kinase activity (EC:2.7.1.30) as compared to a parent gene from which the mutant gene was derived, wherein the amino acid sequence of said mutant polypeptide has at least 80% amino acid sequence identity to SEQ ID No.: 4, and wherein said sequence comprises an E572Q mutation as compared to the polypeptide encoded by the parent gene (where the parent gene encodes an amino acid sequence having at least 80% amino acid sequence identity to SEQ ID No.:2 wherein amino acid residue 572 is E);

and

b. inactivating one or more endogenous genes selected from among a KGD1, KGD2, and LPD1 gene in said yeast, or

modifying an endogenous KGD1 gene in said yeast, wherein the modified gene encodes an amino acid sequence having at least 80% amino acid

sequence identity to SEQ ID No.: 8, and wherein said amino acid sequence comprises an A990D mutation; and

c. inactivating an endogenous UBC13 gene in said yeast, or modifying an endogenous UBC13 gene in said yeast, wherein the modified gene encodes an amino acid sequence of no more than 71 residues and having at least 80% amino acid sequence identity to amino acid residues 1-71 of SEQ ID No.: 14 or 16.

The invention further provides a method for culturing cells of the genetically modified yeast of the invention comprising the steps of:

a. introducing the cells into a defined cultivation medium comprising a carbon source to produce a cell culture,

b. cultivating the cell culture of (a) under aerobic growth conditions;

wherein at least 80% by weight of the carbon source is glycerol.

The invention further provides for the use of the genetically modified yeast of the invention, as a cell factory, where for example, the cell factory is provided with a culture medium comprising glycerol as sole carbon source.

## Description of the invention

### FIGURES

**Figure 1**. Graphical presentation of the cell density (measured as $OD_{600}$ equivalents) of a parental laboratory yeast strain (CEN.PK (CEN.PK113-1A)) and industrial-yeast strains (L.1528 and CLIB382) over time during cultivation on MG medium comprising a defined mineral (M) media supplemented with 10 mL/L of glycerol as sole carbon source.

**Figure 2**. Graphical presentation of the cell density (measured as $OD_{600}$ equivalents) of re-engineered yeast strains, derived from a parental laboratory yeast strain (CEN.PK (CEN.PK113-1A)) and comprising two or three of the mutant genes (GUT1-1; KGD1-1; UBC13-1 and INO80-1) as compared to Evolved ALE#2 yeast strain, when measured over time during cultivation on MG medium comprising a defined mineral (M) media supplemented with 10 mL/L of glycerol as sole carbon source.

**Figure 3**. Graphical presentation of the cell density (measured as $OD_{600}$ equivalents) of re-engineered yeast strains, derived from a parental industrial-yeast strains (L.1528 and CLIB382) and comprising one or all of the mutant genes (GUT1-1; KGD1-1; UBC13-1), as measured over time during cultivation on MG medium comprising a defined mineral (M) media supplemented with 10 mL/L of glycerol as sole carbon source.

**Figure 4.** Graphical presentation of the growth rate the evolved yeast strain ALE#2, and re-engineered yeast strains, derived from a parental laboratory yeast strain (CEN.PK (CEN.PK113-1A)) and comprising two or three of the mutant genes (GUT1-1; KGD1-1; UBC13-1), measured during cultivation on MG medium comprising a defined mineral (M) media supplemented with 10 mL/L of glycerol as sole carbon source. Growth rates were estimated by finding the linear fit with the highest slope of the log(OD values or CDW). Growth analyses were performed in triplicate with the exception of the strain GUT1_UBC13, which was analysed in duplicate.

**Figure 5** Graphical image showing Log2 fold differences (log2FC) in levels of expression and translation of genes in re-engineered yeast strains of the following pairs: (A) GUT1-1; KGD1-1 yeast strain (R-GK), compared to re-engineered GUT1-1; KGD1-1; UBC13-1 yeast strain (R-GKU) and (B) GUT1-1; UBC13-1 yeast strain (R-GU), compared to re-engineered R-GKU yeast strain; based on data derived from transcriptomic and proteomic analysis. Black circles depict only significantly changed transcripts (padj < 0.1), black squares depict only significantly changed levels of proteins (padj < 0.1), and empty diamonds depict significantly altered levels in both protein and transcript (padj < 0.1). Pho3 encodes a constitutively expressed acid phosphatase involved in phosphate metabolism; Cit3 encodes a CITrate synthase having dual mitochondrial citrate and methylcitrate synthase activity; Dld3 encodes a D-Lactate Dehydrogenase (that converts D-2-hydroxyglutarate to alpha-ketoglutarate in the presence of FAD, with concomitant reduction of pyruvate to D-lactate).

**Figure 6** Cartoon showing observed and predicted changes in carbon metabolism in yeast strains of the invention, having gene mutations encoding GUT1-1; KGD1-1; UBC13-1 alleles. Black dashed arrows represent predicted

flux changes when the carbon source for yeast fermentation is shifted from glucose to glycerol. Black dashed lines with white dots and grey boxes depict observed flux changes estimated by comparing measured metabolite ratios in the R-GKU vs. R-GU mutants grown in MG medium. GABA: γ-aminobutyric acid; SSA: succinate semialdehyde.

**Abbreviations and terms:**

**Amino acid sequence identity**: The term "sequence identity" as used herein, indicates a quantitative measure of the degree of homology between two amino acid sequences of substantially equal length. The two sequences to be compared must be aligned to give a best possible fit, by means of the insertion of gaps or alternatively, truncation at the ends of the protein sequences. The sequence identity can be calculated as ((Nref-Ndif)100)/(Nref), wherein Ndif is the total number of non-identical residues in the two sequences when aligned and wherein Nref is the number of residues in one of the sequences. Sequence identity can alternatively be calculated by the BLAST program e.g. the BLASTP program (Pearson W.R and D.J. Lipman (1988)) (www.ncbi.nlm.nih.gov/cgi-bin/BLAST). In one embodiment of the invention, alignment is performed with the sequence alignment method ClustalW with default parameters as described by Thompson J., et al 1994, available at http://www2.ebi.ac.uk/clustalw/.

Preferably, the numbers of substitutions, insertions, additions or deletions of one or more amino acid residues in the polypeptide as compared to its comparator polypeptide is limited, i.e. no more than 1, 2, 3, 4, 5, 6, 7, 8, 9, or 10 substitutions, no more than 1, 2, 3, 4, 5, 6, 7, 8, 9, or 10 insertions, no more than 1, 2, 3, 4, 5, 6, 7, 8, 9, or 10 additions, and no more than 1, 2, 3, 4, 5, 6, 7, 8, 9, or 10 deletions. Preferably the substitutions are conservative amino acid substitutions: limited to exchanges within members of group 1: Glycine, Alanine, Valine, Leucine, Isoleucine; group 2: Serine, Cysteine, Selenocysteine, Threonine, Methionine; group 3: proline; group 4: Phenylalanine, Tyrosine, Tryptophan; Group 5: Aspartate, Glutamate, Asparagine, Glutamine.

**CRISPR system:** is a Clustered Regularly Interspaced Short Palindromic Repeats is a bacterial immune system that has been modified for genome engineering, including the yeast genome.

**Inactivated gene**: the inactivation of a gene from the genome of a microbial cell leads to a loss of function (knockout) of the gene and hence where the gene encodes a polypeptide the inactivation results in a loss of expression of the encoded polypeptide or a failure to express a functional polypeptide. Where the encoded polypeptide is an enzyme, the gene inactivation leads to a loss of detectable enzymatic activity of the respective polypeptide in the microbial cell. An inactivation gene in the genome of a microbial cell is characterized by a loss of function due to the deletion of, or substitution of, or addition of, at least one nucleotide leading to a loss of expression of a polypeptide or failure to express a functional polypeptide encoded by the gene.

**gi number:** (genInfo identifier) is a unique integer which identifies a particular sequence, independent of the database source, which is assigned by NCBI to all sequences processed into Entrez, including nucleotide sequences from DDBJ/EMBL/GenBank, protein sequences from SWISS-PROT, PIR and many others.

**Endogenous gene**: is a gene that originates from within the genome of a micro-organism, and is considered to be a native gene in said micro-organism.

**Detailed description of the invention**

The present invention provides a genetically modified yeast with improved glycerol catabolism, having the ability to grown on a medium comprising glycerol as sole carbon source and without an added source of amino nitrogen. The yeast strains of the invention are genetically engineered such as to be capable of expressing elevated levels of glycerol kinase activity (EC:2.7.1.30); while being unable to express functional enzymes conferring alpha ketoglutarase complex activity and E2 ubiquitin-conjugating activity. Surprisingly, these genetically engineered yeast strains exhibit a growth rate of up to $0.23.h^{-1}$ when grown on minimal medium, with 50g/L glycerol as the sole carbon source, and they exhibited a lag time of only 4 hours (when the starting inoculum had an OD600 of ≥0.3); thereby representing a significantly improved glycerol-growth phenotype over reported genetically-engineered yeast strains.

**I Genotype of a micro-organism of the invention**

The genetically modified yeast is capable of expressing enhanced levels of glycerol kinase activity (EC:2.7.1.30). In one embodiment, the genetically modified yeast comprises a transgene, integrated into the genome, that encodes a polypeptide having glycerol kinase activity (EC:2.7.1.30), thereby enhancing the level of glycerol kinase activity detectable in the cell during growth on glycerol. Preferably the amino acid sequence of the polypeptide has at least 80%, 82, 84%, 86%, 88%, 90%, 92%, 94%, 96%, 98% or 100% amino acid sequence identity to SEQ ID No.: 2. In a second embodiment, the yeast comprises a mutant gene encoding a mutant polypeptide having enhanced glycerol kinase activity (EC:2.7.1.30) as compared to the polypeptide encoded by the parent gene from which the mutant was derived. The amino acid sequence of said glycerol kinase has at least 80%, 82, 84%, 86%, 88%, 90%, 92%, 94%, 96%, 98% or 100% amino acid sequence identity to SEQ ID No.: 4, and wherein said sequence comprises an E572Q mutation with respect to the polypeptide encoded by the parent gene. The mutant gene encoding the glycerol kinase may be a transgene integrated into the genetically modified yeast; or the mutant gene may be a genetically edited endogenous GUT1 gene.

Additionally, the genetically modified yeast is devoid of genes capable of expressing a functional alpha ketoglutarase complex, as well as being devoid of genes capable of expressing a polypeptide having E2 ubiquitin-conjugating enzyme activity (EC:2.3.2.23) (see Example 5.5).

The genetically modified yeast of the invention is not capable of forming a functional alpha ketoglutarase complex due to inactivation of endogenous gene(s) required to express one or more functional polypeptide components of this complex. The complex comprises three subunits: 2-KetoGlutarate Dehydrogenase (Kgd1) (EC:1.2.4.2); Dihydrolipoyl transsuccinylase (Kgd2) (EC 2.3.1.61); and dihydrolipoamide dehydrogenase (Lpd1) (EC:1.8.1.4). The complex catalyzes the oxidative decarboxylation of alpha-ketoglutarate to succinyl-CoA in the TCA cycle, is a key control point in the citric acid cycle. Inactivation of the alpha ketoglutarase complex in the yeast of the invention

is thought to result in an uncoupling of the TCA cycle and oxidative phosphorylation (see Example 5.5).

In one embodiment, the genetically modified yeast of the invention is devoid of a KGD1 gene that is capable of expressing Kgd1; due to the inactivation or deletion of the endogenous KGD1 gene. Preferably the amino acid sequence of the polypeptide has at least 80%, 82, 84%, 86%, 88%, 90%, 92%, 94%, 96%, 98% or 100% amino acid sequence identity to SEQ ID No.: 6. Inactivation of an endogenous KGD1 gene may be due to a failure to transcribe and express the KGD1 gene; or may be due to a failure to express a functional alpha-ketoglutarate dehydrogenase enzyme.

In one embodiment, the genetically modified yeast of the invention comprises a mutant KGD1 gene encoding an amino acid sequence having at least 80%, 82, 84%, 86%, 88%, 90%, 92%, 94%, 96%, 98% or 100% amino acid sequence identity to SEQ ID No.: 8, and wherein said amino acid sequence comprises an A990D mutation when compared to the polypeptide encoded by the parent gene from which the mutant gene was derived. The genetically modified yeast of the invention comprises a mutant KGD1 gene may be derived from a parent yeast strain by means of genetically editing the endogenous KGD1 gene in cells of the parent yeast, as described in Example 3.

In an alternative embodiment, the genetically modified yeast of the invention is devoid of a KGD2 gene that is capable of expressing dihydrolipoyl transsuccinylase (Kgd2) (EC 2.3.1.61); due to the absence of this gene or due to the inactivation of the endogenous KGD2 gene. Preferably the amino acid sequence of the polypeptide has at least 80%, 82, 84%, 86%, 88%, 90%, 92%, 94%, 96%, 98% or 100% amino acid sequence identity to SEQ ID No.:10.

In an additional alternative embodiment, the genetically modified yeast of the invention is devoid of a LPD1 gene that is capable of expressing dihydrolipoamide dehydrogenase (EC:1.8.1.4); due to the absence of this gene or due to the inactivation of the endogenous LPD1 gene. Preferably the amino acid sequence of the polypeptide has at least 80%, 82, 84%, 86%, 88%, 90%, 92%, 94%, 96%, 98% or 100% amino acid sequence identity to SEQ ID No.:12 .

Additionally, the genetically modified yeast of the invention is devoid of a UBC13 gene that is capable of expressing a polypeptide having E2 ubiquitin-conjugating enzyme activity; due to the absence of this gene or due to the inactivation of the endogenous UBC13 gene. Preferably the amino acid sequence of the polypeptide has at least 80%, 82, 84%, 86%, 88%, 90%, 92%, 94%, 96%, 98% or 100% amino acid sequence identity to SEQ ID No.: 14. Inactivation of an endogenous UBC13 gene may be due to a failure to transcribe and express the UBC13 gene; or may be due to a failure to express a functional E2 ubiquitin-conjugating enzyme.

In one embodiment, the genetically modified yeast of the invention comprises a mutant UBC13 gene encoding an amino acid sequence of no more than 71 residues having at least 80%, 82, 84%, 86%, 88%, 90%, 92%, 94%, 96%, 98% or 100% amino acid sequence identity to SEQ ID No.: 16. The genetically modified yeast of the invention comprises a mutant UBC13 gene may be derived from a parent yeast strain by means of genetically edited endogenous UBC13 gene in cells of the parent yeast, as described in Example 3.

The genetically modified yeast of the invention may be selected from a member of the genus *Saccharomyces* (e.g. *S. cerevisiae), Kluyveromyces* (e.g. *K. lactis, K. marxianus* and *Lachancea thermotolerans*); *Komagataella* (e.g. *Komagataella phaffii/ Komagataella pastoris/Pichia pastoris*), *Scheffersomyces* (e.g. *S. stipites/ Pichia stipitis*); *Torulaspora* (*T. delbrueckii*) and *Zygosaccharomyces* (e.g. *Z. bailii and Z. rouxii*). A genetically modified yeast of the invention may be derived from a parent strain selected from any member of the above genera. The respective wild-type GUT1, KGD1 and UBC13 genes and their encoded proteins present in the genome of a parent strain selected from a member of the above genera, are listed in the table below. Genetic modification of the selected parent strain, as taught herein, can be achieved by enhancing expression of the GUT1 gene, and by inactivation of the endogenous KGD1 and UBC13 genes.

**Table 1:** NCBI Accession numbers

| Parent yeast strain | GUT1 | KGD1 | UBC13 |
|---|---|---|---|
| *Kluyveromyces lactis* | NC_006041.1<br>Gene ID: 2893659<br>XP_453973.1 (protein) | NC_006042.1<br>GeneID:2894978<br>XP_455282.1 (protein) | NC_006039.1<br>GeneID:2892070<br>XP_452987.1 (protein) |
| *Kluyveromyces marxianus* | AP012220.1<br>coded_by="AP012220.1:890833..892839"<br>BAO42723.1 (protein) | AP012218.1<br>coded_by="AP012218.1:1154008..1157070"<br>BAO41832.1 (protein) | AP012215.1<br>coded_by="complement(AP012215.1:644825..645307)"<br>BAO39593.1 (protein) |
| *Lachancea thermotolerans* | NC_013081.1<br>GeneID: 8291880<br>XP_002553727.1(protein) | NC_013083.1<br>GeneID:8293712<br>XP_002555435.1 (protein) | NC_013083.1<br>GeneID:8293941<br>XP_002555656.1 (protein) |
| *Komagataella phaffii/ Pichia pastoris* | NC_012966.1<br>GeneID:8200561<br>XP_002494228.1(protein) | NC_012964.1<br>GeneID:8198485<br>XP_002490970.1 (protein) | NC_012964.1<br>GeneID:8198177<br>XP_002490939.1 (protein) |
| *Scheffersomyces stipites* | NC_009048.1<br>GeneID:4840936<br>XP_001386679.2 (protein) | NC_009048.1<br>GeneID:4840878<br>XP_001386444.2 (protein) | NC_009047.1<br>GeneID:4840801<br>XP_001386349.2 (protein) |
| *Torulaspora delbrueckii* | NC_016507.1<br>GeneID: 11504686<br>XP_003682760.1(protein) | NC_016503.1<br>GeneID:11500678<br>XP_003680554.1 (protein) | NC_016501.1<br>GeneID:11502717<br>XP_003678677.1 (protein) |
| Zygosaccharomyces *bailii* | HG316457.1<br>coded_by="complement(HG316457.1:430111..432231)"<br>CDF89478.1(protein) | HG316463.1<br>coded_by="HG316463.1:105237..108287"<br>CDF91165.1(protein) | HG316455.1<br>coded_by="complement(join(HG316455.1:1041073..1041504,HG316455.1:1041563..1041592))"<br>CDF88503.1(protein) |
| Zygosaccharomyces *rouxii* | NC_012996.1<br>GeneID: 8206132<br>XP_002498329.1(protein) | NC_012990.1<br>GeneID:8201500<br>XP_002494434.1(protein) | NC_012991.1<br>GeneID:8202611<br>XP_002495450.1 (protein) |

**II Cultivation of a micro-organism of the invention**

The invention provides a method for culturing cells of the genetically modified yeast comprising the steps of:

a. introducing the cells into a defined cultivation medium comprising a carbon source to produce a cell culture,

b. cultivating the cell culture of (a) under aerobic growth conditions;

wherein at least 80%, 85%, 90%, 95% or 100% by weight of the carbon source is glycerol.

The cultivation medium may comprise 10 – 60 g/L glycerol, for example at least 15, 20, 25, 30, 35, 40, 45, 50, 55 g/L glycerol.

The defined cultivation medium may be a synthetic medium. Preferably the medium comprises a source of minerals, inorganic nitrogen, trace metals and vitamins, as illustrated in Example 1. Additional supplementary carbon sources may include: methanol, molasses (sugar cane of sugar beet), corn syrup and hydrolyzed biomass (e.g. lignocellulose).
According to the method, the cells may be culture by batch fermentation, or in a chemostat under continuous aeration.

**III Use of a micro-organism of the invention as a cell factory**

The invention provides for the use of a genetically modified yeast of the genus *Saccharomyces (e.g. S. cerevisiae), Kluyveromyces (e.g. K. lactis, K. marxianus and Lachancea thermotolerans); Komagataella (e.g. Komagataella phaffii/ Komagataella pastoris/Pichia pastoris), Scheffersomyces (e.g. S. stipites/ Pichia stipitis); Torulaspora (T. delbrueckii) and Zygosaccharomyces (e.g. Z. bailii and Z. rouxii)* with improved glycerol catabolism as a cell factory; where the cell factory is for cultivation in a culture medium comprising glycerol as sole carbon source or at least where glycerol provides 80% of the carbon source.

A genetically modified micro-organism of the invention may be a strain of yeast that is suitable for use as a cell factory for the production of valuable compounds, including biofuels, small chemicals, pharmaceuticals and nutraceuticals. Thus the genetically modified micro-organism of the invention may be derived from a parent yeast strain, where the parent yeast strain itself comprises one or more genetic modification resulting in the expression of genes encoding heterologous single and multi-step metabolic pathways, allowing the production of these compounds. Alternatively, a parent yeast strain may first be genetically modified to produce the micro-organism of the invention; which thereafter is subjected to further genetic modified to introduce metabolic pathways required for the production of valuable compounds.

**IV Methods for producing and identifying a genetically modified yeast of the invention**

The present invention provides a method for enhancing glycerol metabolism in a parent yeast strain by genetic modification of cells of the parent strain to produce a genetically modified yeast of the invention.

A nucleic acid molecule comprising a gene encoding a polypeptide having glycerol kinase activity (EC:2.7.1.30), may be introduced into an integration or self-replicating vector suitable for cloning and introducing into cells of a parent micro-organism using methods and techniques for transformation that are well known to those skilled in the art (see, e.g., Sambrook et al., Molecular Cloning: A Laboratory Manual, Second Edition, Cold Spring Harbor Laboratory Press, 1989).

The existing endogenous GUT1 and UBC13 genes and at least one the alpha ketoglutarase complex genes (KGD1, LPD1 and KGD2), in a cell of a selected parent yeast may be genetically modified to produce a yeast of the invention. For example, the nucleic acid sequence of an endogenous gene can be modified by means of gene editing using CRISPR-Cas9 techniques (1, 2) optimized for the *S. cerevisiae*, as illustrated in Example 3.

The endogenous UBC13 gene and at least one of the endogenous KGD1, LPD1 and KGD2 genes in a cell of a selected parent yeast may be inactivated by deletion of, or substitution of, or addition of, at least one nucleotide within the gene leading to a loss of expression of a functional polypeptide encoded by the gene. Suitable techniques for gene inactivation (knock-out) can be achieved by gene targeting via homologous recombination, techniques that are standard in the art; or by means of gene editing using CRISPR-Cas9 techniques (1, 2). A nucleic acid molecule, that leads to one or more genetic modifications of GUT1, KGD1, LPD1, KGD2 or UBC13 can be introduced into a yeast of interest via well-established genetic transformation techniques (3).

A genetically modified yeast of the invention is characterised by a lack of both alpha ketoglutarase complex activity (e.g. 2-ketoglutarate dehydrogenase enzyme activity (EC: 1.2.4.2)) and E2 ubiquitin-conjugating enzyme activity (EC:2.3.2.23).

A commercially available assay (for example, BioVision, http://www.biovision.com/alpha-ketoglutarate-dehydrogenase-activity-colorimetric-assay-kit-7786.html) or a well-established assay as described in (4) may be used to measure 2-ketoglutarate dehydrogenase enzyme activity (EC: 1.2.4.2) to identify a micro-organism of the invention lacking this enzyme activity. Microorganisms lacking the KGD1 activity can also be identified by analysing growth curves of mutant strains cultured on a minimal glucose media and observing the lack of growth after diauxic shift (deficiency in respiratory growth) (4).

An enzyme assay for measuring E2 ubiquitin-conjugating enzyme activity (EC:2.3.2.23) to identify a micro-organism of the invention lacking this enzyme activity is described in the literature (7).

Additionally, a genetically modified yeast of the invention lacking polypeptides having alpha-ketoglutarate dehydrogenase enzyme activity (EC: 1.2.4.2) and polypeptides having E2 ubiquitin-conjugating enzyme activity (EC:2.3.2.23) can be detected by immunodetection. Preparation of cell extracts and immunodetection methods for detecting the presence of these specific polypeptides are well known in the art.

**EXAMPLES**

**Example 1.** Isolation and characterization of a mutant yeast strain adapted for growth on glycerol

Yeast strains having a modified growth phenotype, having an enhanced ability to grow on glycerol as a carbon source as compared to a parent yeast strain from which they were derived, were developed by adaptive laboratory evolution (ALE). The parent yeast strain was the wild-type laboratory strain CEN.PK113-7D [CBS 8340: http://www.cbs.knaw.nl/Collections/BioloMICS.aspx]. The yeast strains were grown and evolved by ALE by cultivation using the growth media set out below.

**1.1** Cultivation media for isolation of the mutant yeast strain:
The cultivation media were based on a defined mineral (M) media described

by Verduyn et al., (8) containing 5 g/L $(NH_4)_2SO_4$, 3 g/L $KH_2PO_4$, 0.75 g/L $Mg_2SO_4$, 1.5 mL/L trace metal solution and 1.5 mL/L vitamins solution. The composition of the trace metal solution was 3 g/L $FeSO_4 \cdot 7H_2O$, 4.5 g/L $ZnSO_4 \cdot 7H_2O$, 4.5 g/L $CaCl_2 \cdot 6H_2O$, 0.84 g/L $MnCl_2 \cdot 2H2O$, 0.3 g/L $CoCl_2 \cdot 6H_2O$, 0.3 g/L $CuSO_4 \cdot 5H_2O$, 0.4 g/L $NaMoO_4 \cdot 2H_2O$, 1 g/L $H_3BO_3$, 0.1 g/L KI and 15 g/L $Na_2EDTA \cdot 2H_2O$. The vitamin solution includes 50 mg/L d-biotin, 200 mg/L para-amino benzoic acid, 1.0 g/L nicotinic acid, 1.0 g/L Ca-pantothenate, 1.0 g/L pyridoxine-HCl, 1.0 g/L thiamine-HCl and 25 mg/L inositol. The mineral medium, when supplemented with 10 mL/L of glycerol as sole carbon source, was designated "MG" medium. The pH was adjusted with $KOH/H_2SO_4$ to 4.2. When the "MG" medium was additionally supplemented with 1.92 g/L of Y1501 amino acid mix (Sigma), it was designated "MG+" medium. In the case of small-scale cultivation, the cultivation media were filter-sterilized using bottle-top (0.45 µm pore size) filters (VWR; https://us.vwr.com/store/product/4829245/nalgene-rapid-flow-filter-units-and-bottle-top-filters-pes-membrane-sterile-thermo-scientific). In the case of 1L batch fermentation experiments, the cultivation medium was heat-sterilized, sterile vitamin solution and glycerol were added after the medium had cooled to 30°C or less.

**1.2** Isolation of the mutant yeast strain by ALE

The parent strain was pre-cultured in 500 mL shake flask with 50 mL of MG+ medium to provide a starter culture, which was used to inoculate five flat-bottom plastic tubes with 15 mL of MG+ medium at a starting cell density of 0.3 $OD_{600}$. The five tubes were cultured at 30°C with constant agitation using a magnetic stirrer at 1000 rpm. Growth in each tube was monitored, and once the cell culture reached early exponential growth phase, an aliquot of 900 µL was then serially passaged to another tube comprising fresh medium. In order to induce ALE during serial passaging of the cultured cell population, the composition of the growth medium was gradually changed from MG+ to the MG medium (by providing a mixture of the MG+ and MG medium starting with a ratio of 100:0 and gradually transferring to a ratio of 0:100). Yeast cultures were evolved over at least 300 cell generations, resulting in strains capable of growing on a minimal medium supplemented with glycerol as carbon source. One of the best performing strains was designated, ALE#2.

**1.3** Characterization of the mutant yeast strain isolated by ALE

ALE#2 was analysed by whole-genome sequencing that revealed the genetic mutations in relation to the parental strain CEN.PK113-7D. Further genetic characterization, using re-engineering, was performed to pinpoint three causative mutations, located in the genes: GUT1, KGD1 and UBC13, that were found responsible for the observed superior glycerol growth phenotype of ALE#2.

The identified mutant GUT1 gene [SEQ ID No.:3] had a positive strand G1711C substitution as compared to wild-type GUT1, and encoded a mutant Glycerol UTilization polypeptide, Gut1p (>GUT1_1; [SEQ ID No.:4]) having a (E572Q) substitution.

The identified mutant KGD1 gene [SEQ ID No.:7] had a positive strand C2969A substitution as compared to wild-type KGD1, and encoded a mutant 2-KetoGlutarate Dehydrogenase polypeptide, Kgd1p (>KGD1_1; [SEQ ID No.:8]) having a (A990D) substitution.

The identified mutant UBC13 gene [SEQ ID No.:15] had a positive strand 209ΔG deletion as compared to wild-type UBC13, creating a frame shift, and encoded a C-terminally truncated mutant UBiquitin-Conjugating polypeptide, Ubc13 (>UBC13; [SEQ ID No.:16]) having an R70L substitution and a stop codon after the 71$^{st}$ a.a. residue.

Additionally, ALE#2 was found to contain a point mutation in the INO80 gene. The mutant INO80 gene [SEQ ID No.:17] had a positive strand G1076A substitution encoding a mutant ATPase and nucleosome spacing factor (EC: 3.6.4.12), INO80_1 [SEQ ID No.:18]) having a C359Y substitution.

**Example 2**. Functional properties of the polypeptides encoded by the mutant genes in the adapted yeast strain, ALE#2

The enzyme, glycerol kinase (Gut1) phosphorylates glycerol to glycerol-3-phospate as the initial step of glycerol catabolism. The presence of the Gut1 enzyme in yeast is indispensable for its utilization of glycerol, and hence deletion of the GUT1 gene in yeast completely abolishes growth on glycerol. The mutant GUT1 gene, comprising the SNP (G1711C), encodes a mutant glycerol kinase Gut1 having an E572Q substitution. The mutation confers a

gain-of-function phenotype which improves flux in the glycerol uptake pathway resulting in an increased efficiency of glycerol catabolism (figure 2). While not wishing to be bound by theory, *in silico* structural analyses indicate that this gain-of-function may be attributed to improved ATP binding efficiency, since the E572Q lies in close proximity to the ATP binding (separated by only 12 amino acid residues). In addition, the E572Q may serve to stabilise enzyme tetramer formation, or impart insensitivity towards allosteric inhibition.

KGD1 is a component of a mitochondrial multiprotein alpha ketoglutarase complex, comprising three subunits: 2-KetoGlutarate Dehydrogenase (Kgd1); Dihydrolipoyl transsuccinylase (Kgd2); and dihydrolipoamide dehydrogenase (Lpd1). The complex catalyzes the oxidative decarboxylation of alpha-ketoglutarate to succinyl-CoA in the TCA cycle, and is a key control point in the citric acid cycle. The mutant KGD1 gene, comprising the SNP (C2969A), encodes a mutant Kgd1p having an A990D substitution. While not wishing to be bound by theory, *in silico* modelling studies indicate that the mutation structurally disables the assembly of the alpha ketoglutarase complex, and thereby slows flux through the citric acid cycle. This accounts for the loss of alpha ketoglutarase activity in strains carrying the KGD1-1 allele; and an inability to grow on ethanol (characteristic for *kgd1* deletion mutants).

UBC13 is an E2 ubiquitin-conjugating polypeptide, performing the second step in the ubiquitination reaction that targets a protein for degradation via the proteasome; and involved in the error-free DNA post-replication repair pathway. The mutant UBC13 gene, comprising a deletion (209ΔG) that creates a frame shift, encodes a C-terminally truncated mutant UBiquitin-Conjugating polypeptide of 71 amino acids, having a R70L substitution. The truncated polypeptide lacks the active site cysteine residue required to form a thiol-ester with ubiquitin or ubiquitin-like proteins. While not wishing to be bound by theory, it is predicted that inactivation of the UBC polypeptide due to its truncation, confers a gain-of-function phenotype which increases the efficiency of glycerol catabolism.

**Example 3**. Re-engineering yeast strains adapted for growth on glycerol

2.1 Reengineering mutations in wild-type yeast strains

The mutations identified in the three genes (GUT1_1, KGD1_1 and UBC13_1) were introduced into selected wild type yeast strains by means of CRISPR-Cas9 techniques (1, 2) optimized for *S. cerevisiae.* A specific synthetic guide RNA (gRNA) sequence (see Table 2) was designed for targeting the Cas9 nuclease to the appropriate genetic locus at which each genetic modification was to be introduced. The quality and specificity of gRNA was assessed using a *CRISPRdirect* online tool developed by Naito et al., (9). Repair templates (90-bp dsOligo flanking 45 bp up- and 45 bp downstream of the specific cut site) were designed to repair the DNA double strand break introduced by the Cas9 nuclease at each genetic locus. Each repair template contained a specific mutation and a silent mutation that would disturb the Protospacer Adjacent Motif site, PAM (5'-NGG-3') (see Table 2).

**Table 2:** List of oligonucleotides

| Target | Sequences displayed in 5' to 3' direction | SEQ ID No. |
|---|---|---|
| | **Guide RNA sequence (underlined)** | |
| *PPZ2_1* | CCAATGTTACAAGAGTCTACGTTTTAGAGCTAGAA | 19 |
| *GUT1_1* | CATCCACTGCCAGAACCGACGTTTTAGAGCTAGAA | 20 |
| *KGD1_1* | CgGCAGCAACAgCACCACTTGTTTTAGAGCTAGAA | 21 |
| *UBC13_1* | TATATCATCCCAATATTGATGTTTTAGAGCTAGAAA TAGCAAG | 22 |
| *INO80_1* | GGAATCGATTGGATTGTAGTGTTTTAGAGCTAGAA ATAGCAAG | 23 |
| | ***Double stranded repair templates (only positive strand shown)*** | |
| *GUT1_1 repair* | AGACAGGGACTTTTTAGAGGAAATTTCCGACGTCA CATATcAAAAGTCGCCaCTGTCGGTTCTGGCgGTGG ATGGCGGGATGTCGAGGTC | 24 |
| *KGD1 repair* | TAAGGATTTCAAGGTCAGATACTGTGGTAGAAAtCC AAGTGGTGaTGTTGCTGCaGGTAGCAAATCACTAC ATTTGGCCGAAGAAGATGC | 25 |
| *UBC13 repair* | ACGACTATCCAATGGAAGCACCGAAAGTACTTTTTT AACCAAGATATATCATCCCAATATTGATAGaTTGGG CCGTATATGTCTTGATGT | 26 |
| *INO80 repair* | CAAGCATCAGAAGATTAAGAAGAGGAGGTTCACCA ATTaTATTGTCACaGACTACAATCCAATCGATTCCA AGTTGAACATCAAAATAAC | 27 |
| | ***Primers for validation*** | |
| *GUT1F* | TAGTCAAGAGAAACCTGCCC | 28 |
| *GUT1R* | ACCTTCTGACTTTGACACAG | 29 |
| *KGD1F* | ACCCAAGATATTTCCCATCTG | 30 |
| *KGD1R* | CATCTTTAGGATTGTTGGAAAAC | 31 |
| *UBC13F* | AGTAAGTGACCCAGTACCTGGC | 32 |
| *UBC13R* | TCACTCGGGTTTCTTCTTTGC | 33 |
| *INO80F* | AGAACAGGATGACAATGACGA | 34 |
| *INO80R* | CAACCCGTGTCTAGTGTTG | 35 |

Cells of four parental strains (Laboratory strain CEN.PK113-1A (isogenic to the CEN.PK113-7D) and industrial strains (CLIB328 and L1528) listed in Table 3) were each transformed with the Cas9 expressing plasmid pCfB2312 (2) resulting in the respective transformed strains (e.g. reconstructed laboratory strain 1A_Cas9). The yeast transformants were selected on yeast extract peptone dextrose (YPD) agar plates supplemented with the selective antibiotic, Geneticin (G418). Subsequently, cells of each Cas9 expressing strain were individually transformed with different plasmids expressing a single gRNA used to introduce a point mutation in one of the selected genetic loci (see Table 2). The resulting transformed Cas9 cells were selected on YPD agar plates supplemented with the selective antibiotics G418 and Nourseothricin (CloNat).

In order to confirm that the gene mutagenesis using CRISPR-Cas9 had introduced the intended mutations, five yeast colonies of each of the selected yeast strains was tested as follows: Colony-PCR, using OneTaq® 2X Master Mix (New England Biolabs) and the validation primers listed in Table 2, were used to amplify a 500 bp long DNA fragment flanking the mutated locus, which was then column purified using NucleoSpin® kit (MACHEREY-NAGEL) and sent for sequencing (Eurofins Genomics). Each engineered strain harboring a correct gene mutation was streaked on YPD+G418 agar plates supplemented, and incubated for 2-3 days at 30°C. Subsequently, yeast strains were replica-plated on YPD+G418+CloNat and YPD+G418 media in order to select for the mutants that have lost the respective gRNA expressing plasmid.

Yeast cells with a single gene mutation (lacking the corresponding gRNA plasmid) were then transformed with an additional gRNA expressing plasmid targeting a different locus. Subsequently, gRNA plasmids were "kicked out" from cells of the selected strain harboring two gene mutations. The CRISPR-Cas9 gene mutagenesis procedure was then repeated on the selected strains, in order to generate yeast strains containing three gene mutations (see Table 3).

**Table 3**. List of the yeast strains

| Name | Genotype |
|---|---|
| ***PARENTAL*** | |
| CEN.PK113-7D (10) | *MATa MAL2-8c SUC2* |
| CEN.PK113-1A (11) | *MATα MAL2-8c SUC2* |
| CLIB328 | *MATα/ MATa* wild type diploid industrial strain |
| L1528 | *MATα/ MATa* wild type diploid industrial strain |
| ***EVOLVED*** | |
| ALE#2 | *MATa MAL2-8c SUC2 evolved* |
| ***RECONSTRUCTED*** | |
| 1A_Cas9 | *MATα MAL2-8c SUC2 pCfB2312::KanMX* |
| GUT1_1 | *MATα MAL2-8c SUC2 GUT1(E572Q)* |
| KGD1_1 | *MATα MAL2-8C SUC2 KGD1(A990D)* |
| UBC13_1 | *MATa MAL2-8C SUC2 UBC13(R70fs)* |
| GUT1_INO80 | *MATα MAL2-8c SUC2 GUT1(E572Q) INO80(C359Y)* |
| GUT1_UBC13 | *MATα MAL2-8c SUC2 GUT1(E572Q) UBC13(R70fs)* |
| GUT1_KGD1 | *MATα MAL2-8c SUC2 GUT1(E572Q) KGD1(A990D)* |
| UBC13_INO80 | *MATa MAL2-8C SUC2 UBC13(R70fs) INO80(C359Y)* |
| KGD1_INO80 | *MATα MAL2-8C SUC2 KGD1(A990D) INO80(C359Y)* |
| GUT1_INO80_KGD1 | *MATα MAL2-8c SUC2 GUT1(E572Q) INO80(C359Y) KGD1(A990D)* |
| GUT1_INO80_UBC13 | *MATα MAL2-8c SUC2 GUT1(E572Q) INO80(C359Y) UBC13(R70fs)* |
| GUT1_UBC13_KGD1 | *MATα MAL2-8c SUC2 GUT1(E572Q) UBC13(R70fs) KGD1(A990D)* |
| ***INDUSTRIAL STRAINS*** | |
| CLIB328_UBC13 | *MATα/MATa UBC13(R70fs)* |
| CLIB328_UBC13_KGD1_UBC13 | *MATα/MATa GUT1(E572Q) UBC13(R70fs) KGD1(A990D)* |
| L1528_UBC13 | *MATα/MATa UBC13(R70fs)* |
| L1528_UBC13_KGD1_UBC13 | *MATα/MATa GUT1(E572Q) UBC13(R70fs) KGD1(A990D)* |

**Example 4**. Growth of evolved and reengineered yeast strains on glycerol

**4.1** Growth performance assessed by micro-scale cultivation

Growth on glycerol of the evolved and reengineered yeast strains, listed in Table 3, was characterized on a microtiter scale using the Growth Profiler 1152 systems (Enzyscreen). An overnight pre-culture was prepared by inoculating each strain into a well of a 24 deep-well plate (Porvair Sciences) filled with 3 mL of YPD medium and incubating at 30°C with 300 rpm shaking. Next, the plate with the pre-culture was centrifuged at 2200g for 5 min and each cell pellet was re-suspended in 3 mL of MG medium (see example 1.1). A 200 µL aliquot of each re-suspended pre-culture was transferred to an

appropriate volume of fresh MG medium such as to give a pre-inoculum suspension having a cell density of 4.5 $OD_{600}$. Finally, 50 µL of each pre-inoculum suspension was inoculated into a separate well of a Krystal 24-well clear bottom white microplate (Porvair Sciences) prefilled with 700 µL MG media per well; and incubated for minimum of 80 hours at 30°C with 225 rpm shaking. Cell growth (green color intensity, G-value) was monitored by scanning the bottom of the plates at 30 minute intervals. G-value was converted to an $OD_{600}$ equivalent by using a calibration curve using a best-fit model of G-value to $OD_{600}$.

The parental laboratory yeast strain (CEN.PK (CEN.PK113-1A)) failed to grow when cultivated for a period of 100h on MG medium comprising a defined mineral (M) media supplemented with 10 mL/L of glycerol as sole carbon source (Figure 1). The parental industrial-yeast strains (L.1528 and CLIB382), showed a limited capacity to grow on the MG medium, after a prolonged lag phase of >80h (Figure 1).

In contrast to the parental strains, the re-engineered yeast strains derived from a parental laboratory yeast strain (CEN.PK (CEN.PK113-1A)), and comprising at least two mutant gene (GUT1-1; KGD1-1; UBC13-1 and INO80-1), showed measurable growth on MG medium comprising a defined mineral (M) media supplemented with 10 mL/L of glycerol as sole carbon source (Figure 2). Growth of the engineered strains was detected after a shorter lag phase, and the cell density reached within 100h was significantly increased. Growth of re-engineered strains comprising three mutant genes was greater than for any combination of two mutant genes. Yeast strains having a combination of the three mutant genes, GUT1-1; KGD1-1; UBC13-1, had a growth rate of $0.21.h^{-1}$ (or $0.23.h^{-1}$ when grown on MG with 50g/L glycerol), and the shortest lag time of 4 hours (when starting inoculum had an $OD_{600}$ of at least 0.3); representing the strongest glycerol-growth phenotype among the re-engineered strains.

Similarly, the re-engineered yeast strains derived from the industrial yeast strains (L.1528 and CLIB382), and comprising at least one mutant gene (UBC13-1) showed improved growth on MG medium (Figure 3). Growth of the engineered strains was detected after a shorter lag phase, and the cell density reached within 100h was significantly increased. In particular, growth of re-

engineered strains comprising three mutant genes GUT1-1; KGD1-1; UBC13-1, showed the strongest glycerol-growth phenotype, in respect of cell density, growth rate and a significantly shorter lag phase.

**Table 3.** Growth of evolved and reengineered yeast strains on glycerol

| Strain background | Genotype | Lag phase | Growth rate, (1/h) |
|---|---|---|---|
| CEN.PK lab | ALE2 evolved | Short (4-20 hours) | 0.237±0.031 |
| CEN.PK lab | GUT1_1, KGD1_1 | Long (80 +hours) | 0.189±0.054 |
| CEN.PK lab | GUT1_1, UBC13_1 | Medium (60 -80 hours) | 0.077±0.016 |
| CEN.PK lab | KGD1_1, UBC13_1 | Medium (60 -80 hours) | 0.122±0.019 |
| CEN.PK lab | KGD1_1, INO80_1 | Medium (60 -80 hours) | 0.225±0.01 |
| CEN.PK lab | GUT1_1, INO80_1, UBC13_1 | Medium (40 -60 hours) | 0.096±0.030 |
| CEN.PK lab | GUT1_1, INO80_1, KGD_1 | Medium (40 -60 hours) | 0.152±0.034 |
| CEN.PK lab | GUT1_1, KGD1_1, UBC13_1 | Short (4-20 hours) | 0.207±0.022 |
| CEN.PK lab* | GUT1_1, KGD1_1, UBC13_1 | Short (4-20 hours) | 0.231±0.020 |
| L1528 ind | WT | Long (100 + hours) | 0.22±0.02 |
| L1528 ind | UBC13_1 | Medium (51-67 hours) | 0.21±0.01 |
| L1528 ind | GUT1_1, KGD1_1, UBC13_1 | Medium (43-50 hours) | 0.24±0.02 |

| | | | |
|---|---|---|---|
| CLIB382 ind | WT | Long (100 + hours) | 0.15 |
| CLIB382 ind | UBC13_1 | Medium (46-60 hours) | 0.14±0.01 |
| CLIB382 ind | GUT1_1, KGD1_1, UBC13_1 | Medium (37-43 hours) | 0.15±0.01 |

*Growth on MG medium comprised 50 g/L glycerol

**4.2** Growth performance assessed by controlled batch fermentation

Growth of the evolved and reengineered yeast strains, listed in Table 3, on glycerol was characterized under batch fermentation conditions as follows. Pre-cultures of the evolved strain ALE#2 and re-engineered strains GUT1_UBC13, GUT1_KGD1 and GUT1_UBC13_KGD1 were prepared by inoculating cells of each strain into 0.5 L shake flasks with 50 mL of MG (pH 4.2), and incubating them in an orbital shaker set to 200 rpm at 30 °C until late-exponential phase ($OD_{600}$ 5 ~ 7). The cell density of the pre-cultures was increased by centrifugation and re-suspending the cells in fresh MG medium, which were used for subsequent batch culture. Batch cultivations were performed under aerobic conditions in one liter Sartorius fermenters equipped with continuous data acquisition (Braun Biotech International). Each fermenter was inoculated to an initial $OD_{600}$ of 0.2. Cell culture aeration was ensured by constant airflow of 2.0 v.v.m. (120 L/h) and a stirring speed of 1000 rpm. The temperature was maintained at 30 °C during the fermentation and pH (4.2) level was controlled by automatic addition of 2M NaOH solution. The exhaust gas composition was constantly monitored by an off-gas analyzer 1311 Fast response triple gas (Innova) combined with Mass Spectrometer Prima Pro Process MS (ThermoFisher Scientific).

The batch cultures were sampled at regular intervals to determine cell density ($OD_{600}$), cell dry weight (CDW).

Cell dry weight (CDW) sampling was performed to determine the biomass concentration of each yeast culture over time. Polyethersulfone filters having a pore size of 0.45 µm Montamil® (Membrane Solutions, LLC), that were used to harvest cells, were pre-dried in a microwave oven at 150 W for 20 min and weighted on an analytical scale. A 5 mL aliquot of each culture was harvested

by filtration through a filter, which was then washed with three volumes of distilled water. Thereafter, the filters with yeast biomass were dried in the microwave oven at 150 W for 20 min and cooled down in a desiccator for a minimum of 2 hours. The filters with dried biomass were weighed in order to determine the CDW.

The growth rate of the evolved yeast strain (ALE#2) cultivated under batch fermentation conditions with 10g/L glycerol, was $0.147\pm0.027.h^{-1}$, which was measurably faster than that of the re-engineered yeast strains, derived from a parental laboratory yeast strain (CEN.PK (CEN.PK113-1A)), comprising two mutant genes, either GUT1-1 and KGD1-1 ($0.094\pm0.011.h^{-1}$) or GUT1-1 and UBC13-1 ($0.107\pm0.005.h^{-1}$) (Figure 4). Surprisingly, the growth rate of the re-engineered yeast strain comprising the three mutant genes: GUT1-1; KGD1-1 and UBC13-1, was $0.130\pm0.007.h^{-1}$, which was not significantly less than the evolved strain ALE#2 (Figure 4). This indicates that the combination of GUT1-1; KGD1-1 and UBC13-1 mutations represent essential genetic modifications required for conferring the glycerol-growth phenotype of the ALE#2 yeast strain.

**Example 5**. Molecular characterization of the re-engineering yeast strains adapted for growth on glycerol

Transcriptomic and proteomic profiles of the re-engineered strains (as described in Example 3) grown in well-controlled reactors, were used to determine the impact of the causal mutant genes (KGD1-1 and UBC13-1) at a molecular level. These data were complemented with an analysis of changes in metabolic flux in the re-engineered strains; in particular to determine the impact of the KGD1-1 mutant gene.

5.1 Transcriptomic analysis procedure

All RNA samples were prepared as follows, 10 mL of fermentation broth, derived from cultivation of each respective yeast strain, was sprayed into 50 mL 25 Falcon® tube filled with ice and immediately centrifuged at 10000xg for 5 min at 4°C. After centrifugation, the supernatant was discarded and the cell pellet was frozen; and kept at -80°C until extraction. Total RNA in each frozen cell pellet was isolated using RNAeasy kit (Qiagen) by following manufacturer's recommendations. Briefly, 594 µL of RLT buffer plus 6µL of β-

mercaptoethanol were added to the Falcon® tube containing the frozen cell pellet and allowed to thaw and the resulting cell suspension was transferred to an ice-cold FastPrep Cap tube containing 600µl of glass beads (400nm acid washed, Sigma) and the cells were then disrupted on ice. Cell lysate was recovered by microcentrifugation (Eppendorf), and the supernatant was careful mixed with 1 volume of 70% HPLC-grade ethanol. Sample was transferred to an RNAeasy column (www.qiagen.com) and washed according the manufacturer's instructions. RNA was then eluted with 60 µL of RNase-free water; digested with Turbo DNAse (Invitrogen Ambion) accordingly to manufacture instructions followed by RNA clean-up (RNAeasy kit, Qiagen). RNA library was prepared using the Illumina TruSeq Stranded mRNA LT sample prep kit starting with 500ng of total RNA, following manufactures instructions using Beckman Biomek FX Laboratory automation station. Samples were sequences using Hiseq2000 instruments in the 50 bp single read mode and loaded 8pM onto the flowcell at the Genomics Core Facility of the EMBL (Heidelberg, Germany).

The quality of the raw RNA sequencing reads was assessed using FastQC (version 0.11.3). Prior to the alignment, adapter trimming was performed using cutadapt (version 1.9.1) with default options providing the standard lllumina TrueSeq Index adapters. Subsequent quality trimming and filtering was performed with FaQCs (version 1.34) using the following parameters: -q 20 -min_L 25 –n 5 -discard 1. The total reads per sample after trimming and filtering ranged from 17.5 to 27 million. The sequencing reads were aligned to the reference genome of *S. cerevisiae* CEN.PK113-7D using tophat2 (version 2.0.10) with the following parameter: -G -T -x 20 –M --microexon-search --no-coverage-search --no-novel-juncs -a 6. Only reads with unique mappings were considered for differential expression analysis. Gene level count tables were obtained using the count script of the HTSeq python library (version 0.6.1p1.) with default options. All reads mapped in total to about 5400 genes. The following statistical analysis was performed using the Bioconductor package DESeq2 (version 1.12.4). Size-factor based normalization to control for batch effects and 1 inter-sample variability and dispersion estimation were conducted using the package defaults. The differential expression analysis was again performed with the package defaults, which include multiple testing correction, independent filtering and cooks cutoff for outlier. Raw P-values as

returned by DESeq2 were used as input to fdrtool (version 1.2.15) in order to compute q-values (false discovery rates (FDRs)). Genes with a FDR < 0.1 were considered as significantly differentially expressed. Biostatistical analyses were conducted using R V.3.3.1 (R Development Core Team).

5.2 Proteomic analysis procedure

For proteomics analysis 10 mL of fermentation broth derived from cultivation of each respective yeast strain was transferred into ice-cold 15 mL Falcon® and immediately centrifuged at 10000xg for 2 min at 4°C. After centrifugation supernatant was discarded and cell pellet was washed once with PBS buffer. Pellet was frozen by placing the tube into dry-ice bath. Frozen samples were kept at -80°C until extraction. Cell pellets were lysed using 0.1% RapiGest in 100 mM ammonium bicarbonate. Three cycles of sonication (Cell disruptor, Sonifier, Branson) were applied to the lysate (1 cycle: 15 seconds sonication, 15 seconds on ice), followed by 15 minutes bead beating using Precellys Lysing Kit (KT0361-1-004.2). Cell lysate was transferred into a new tube after centrifugation (5 minutes, 5000 x g) and incubated at 80°C for 15 minutes. Benzonase (25U, Merck) was added to the lysate for 30 minutes at 37°C. Cysteines were reduced using dithiothreitol (56 °C, 30 minutes, 10 mM). The sample was cooled to 24 °C and alkylated with iodacetamide (room temperature, in the dark, 30 minutes, 10 mM). Proteins were TCA precipitated; TCA pellet was washed by acetone and dried. The proteins were digested in 50 mM HEPES (pH 8.5) using LysC (Wako) with an enzyme to protein ration 1:50 at 37°C for 4 hours, followed by trypsin (Promega) with an enzyme to protein ratio 1:50 at 37°C overnight. TMT10plex™ Isobaric Label Reagent (ThermoFisher) was added to the samples according the manufacturer's instructions. Labeled peptides were cleaned up using OASIS® HLB µElution Plate (Waters).

Offline high pH reverse phase fractionation was performed using an Agilent 1200 Infinity high-25 performance liquid chromatography (HPLC) system, equipped with a Gemini C18 column (3 µm, 110 Å, 100 x 1.0 mm, Phenomenex). The solvent system consisted of 20 mM ammonium formate (pH 10.0) as mobile phase (A) and 100% acetonitrile as mobile phase (B).

Peptides were separated using the UltiMate 3000 RSLC nano LC system (Dionex) fitted with a trapping cartridge (µ-Precolumn C18 PepMap 100, 5µm, 300 µm i.d. x 5 mm, 100 Å) and an analytical column (Acclaim PepMap 100 75 µm x 50 cm C18, 3 µm, 100 Å). The isolated peptides were analysed by LC-MS/MS using a mass spectrometer (QExactive plus, ThermoFisher).

5.3 Detection of metabolic flux in the re-engineered strains

For intracellular metabolomics analysis, cells of each respective yeast strain, cultured on MG medium, were harvested using a modified fast filtration protocol (13), as follows. Briefly, 5ml of culture were sampled at mid-exponential growth phase and were vacuum-filtered through nylon membrane filters (0.45 µm, WhatmanTM), followed by three rapid washing steps with 5 ml of PBS to ensure no contamination from extracellular metabolites. The polar metabolites were extracted by adding the cell-containing filter in 5 ml of cold (-20°C) HPLC-grade 2 methanol (Biosolve Chimie, France)/MilliQ water (1:1, v/v) and incubating for 1h at -20°C. The mixture of metabolites and cell debris was centrifuged at 10000 rpm and 0°C for 10 min, and the supernatants were collected and dried with speed-vac. The dried metabolites were derivatized to their (MeOx)TMS-derivatives through reaction with 100 µL of 20 mg/mL methoxyamine hydrochloride (Alfa Aesar, UK) solution in pyridine (Sigma-Aldrich) for 90 min at 40°C, followed by reaction with 200 µL N-methyl-trimethylsilyl-trifluoroacetamide (MSTFA) (Alfa Aesar, UK) for 10 hours at room temperature. The metabolic profile of each sample was measured thrice using a Shimadzu TQ8040 GC-(triple quadrupole) MS system (Shimadzu Corp.). The metabolite quantification was carried out by calculating the peak areas of the identified marker ions of each metabolite. For glucose, the smaller of the two derivative peaks was used for quantification.

5.4 Modelling of metabolic flux in the re-engineered strains

Metabolic reactions in *S. cerevisiae* that might facilitate glycerol utilization were investigated by means of model simulations. Specifically, a mixed-integer linear programming routine was used to identify a minimum number of reactions, in the genome-scale metabolic model of *S. cerevisiae* (iFF70816) that might be required for optimal glycerol utilization when provided with

glycerol as carbon source. The metabolic flux during respiratory growth on glucose was used as a reference metabolic state. A sub-set of reactions were then identified whose (absolute) flux for optimal glycerol utilization changed by >25 % beyond the (absolute) flux range extremes (12) (± 0.001 mmol/(g CDW h) when 6 C-moles of carbon source were converted to biomass) under the reference metabolic state. Equal C-molar conversion of carbon source to biomass was considered in the reference metabolic state and glycerol utilization.

5.5 Comparative transcriptomic and proteomic profiles combined with metabolic flux analysis reveals the molecular mechanisms underlying the glycerol phenotype observed in the re-engineered strains.

Comparison of the transcriptomic and proteomic profiles of re-engineered *GUT1-1; KGD1-1* yeast strain (*R-GK*), compared to re-engineered *GUT1-1; KGD1-1; UBC13-1* yeast strain (*R-GKU*), showed that the *UBC13-1* mutation only caused minor gene expression differences (Figure 5 A); while Ubc13 protein levels were noticeably lowered in the *R-GKU* strain. This is consistent with a loss-of-function phenotype due to expression of an inactive truncated Ubc13 protein, which was predicted *in silico* for this *UBC13-1* gene mutation. The beneficial role of the *UBC13-1* allele, in enabling the glycerol phenotype, however points to a yet undiscovered function of Ubc13.

Comparison of the transcriptomic and proteomic profiles of re-engineered *GUT1-1; UBC13-1* yeast strain (*R-GU*), compared to re-engineered *R-GKU* yeast strain, showed that the *KGD1-1* allele specifically impacted those cellular processes that are closely linked to the TCA cycle. The *R-GKU* strain is characterised by a decreased expression of Cit3 (citrate synthase, an initial TCA cycle enzyme) as well as Dld3 (2-hydroxyglutarate transhydrogenase), as seen in Figure 5B.

The mechanism whereby the loss-of-function *KGD1* mutation results in improved glycerol metabolism was analysed by comparing flux distributions (including flux variability) detected in re-engineered *R-GKU* yeast cells versus *R-GU* yeast cells; supplemented with data derived from genome-scale metabolic flux modelling of *S. cerevisiae*. Optimal growth on glycerol (with minimum changes to glucose utilization) was observed to depend on the

down-regulation of Kgd1 flux concomitant with up-regulation of oxidative phosphorylation. This implies that the TCA cycle flux and oxidative phosphorylation are uncoupled in the yeast cells of the invention; consistent with decreased expression of the Cit3 TCA cycle enzyme and Dld3. An increased flux through the GABA shunt is further predicted

**References**

1. Jakočiūnas T, et al. (2015) Multiplex metabolic pathway engineering using CRISPR/Cas9 in Saccharomyces cerevisiae. *Metab Eng* 28:213–222.

2. Stovicek V, Borodina I, Forster J (2015) *CRISPR–Cas system enables fast and simple genome editing of industrial Saccharomyces cerevisiae strains* doi:10.1016/j.meteno.2015.03.001.

3. Gietz RD, Schiestl RH (2007) High-efficiency yeast transformation using the LiAc / SS carrier DNA / PEG method. *Nat Protoc* 2(1):35–37.

4. Repetto B, Tzagoloff A (1989) Structure and regulation of KGD1, the structural gene for yeast alpha-ketoglutarate dehydrogenase. *Mol Cell Biol* 9(6):2695–705.

5. Swinnen S, Ho P-W, Klein M, Nevoigt E (2016) Genetic determinants for enhanced glycerol growth of Saccharomyces cerevisiae. Metabolic Engineering 36:68-79

6 Hoek PV, Van Dijken P, and Pronk JT (1998) Effect of Specific Growth Rate on Fermentative Capacity of Baker's Yeast. Appl Environ Microbiol 64(11): 4226-

7. Berndsen CE, Wolberger C (2011) A spectrophotometric assay for conjugation of ubiquitin and ubiquitin-like proteins. *Anal Biochem* 418(1):102–10.

8. Verduyn C, Postma E, Scheffers W a, Van Dijken JP (1992) Effect of benzoic acid on metabolic fluxes in yeasts: a continuous-culture study on the regulation of respiration and alcoholic fermentation. *Yeast* 8(7):501–17.

9. Naito Y, Hino K, Bono H, Ui-Tei K (2015) CRISPRdirect: software for designing CRISPR/Cas guide RNA with reduced off-target sites. *Bioinformatics* 31(7):1120–3.

10. Nijkamp JF et al., (2012) De novo sequencing, assembly and analysis of the genome of the laboratory strain Saccharomyces cerevisiae CEN.PK113-7D, a

model for modern industrial biotechnology. Microbial Cell Factories DOI: 10.1186/1475-2859-11-36

11. Pinel D et al., (2011) Saccharomyces cerevisiae Genome Shuffling through Recursive Population Mating Leads to Improved Tolerance to Spent Sulfite LiquorAppl. Environ. Microbiol. 77(14): 4736-4743

12. Mahadevan, R. & Schilling, C. H. (2003) The effects of alternate optimal solutions in constraint-based genome-scale metabolic models. Metab. Eng. 5, 264–276.

13. Kim, S. et al. (2013) Evaluation and optimization of metabolome sample preparation methods for Saccharomyces cerevisiae. Anal. Chem. 85, 2169–2176.

**Claims**

1. A genetically modified yeast with improved glycerol catabolism, wherein the yeast comprises:
    a. a transgene gene encoding a polypeptide having glycerol kinase activity (EC:2.7.1.30), or
    a mutant gene encoding a mutant polypeptide having enhanced glycerol kinase activity (EC:2.7.1.30) as compared to a parent gene, wherein the amino acid sequence of said mutant polypeptide has at least 80% amino acid sequence identity to SEQ ID No.: 2, and wherein amino acid residue 572 is Q;

    and wherein said yeast is devoid of:

    b. one or more genes required for expressing a functional alpha ketoglutarase complex, selected from the group consisting of:
        i. a gene encoding a polypeptide having alpha-ketoglutarate dehydrogenase enzyme activity (EC: 1.2.4.2);
        ii. a gene encoding a polypeptide having dihydrolipoyl transsuccinylase activity (EC 2.3.1.61); and
        iii. a gene encoding a polypeptide having dihydrolipoamide dehydrogenase (Lpd1) activity (EC:1.8.1.4)
        and
    c. a gene encoding a polypeptide having E2 ubiquitin-conjugating enzyme activity (EC:2.3.2.23).

2. The genetically modified yeast of claim 1, wherein the yeast is a species of a genus selected from among *Saccharomyces, Kluyveromyces, Komagataella, Scheffersomyces, Torulaspora* and *Zygosaccharomyces*.

3. The genetically modified yeast of claim 1 or 2, wherein said one or more genes required for expressing a functional alpha ketoglutarase complex are selected from the group:
    a. KGD1 gene encoding a polypeptide having alpha-ketoglutarate dehydrogenase activity (EC:2.7.1.30);

b. KGD2 gene encoding a polypeptide having dihydrolipoyl transsuccinylase (Kgd2) activity (EC 2.3.1.61), and

c. LPD1 gene encoding a polypeptide having dihydrolipoamide dehydrogenase activity (EC:1.8.1.4),

wherein said one or more genes are inactivated.

4. The genetically modified yeast of claim 3, wherein:

a. the KGD1 gene encodes an amino acid sequence having at least 80% amino acid sequence identity to SEQ ID No.: 6,

b. the KGD2 gene encodes an amino acid sequence having at least 80% amino acid sequence identity to SEQ ID No.: 10, and

c. the LPD1 gene encodes an amino acid sequence having at least 80% amino acid sequence identity to SEQ ID No.: 12.

5. The genetically modified yeast of any one of claims 1 to 3, wherein said yeast comprises a mutant KGD1 gene encoding a mutant polypeptide, wherein the amino acid sequence of the mutant polypeptide has at least 80% amino acid sequence identity to SEQ ID No.: 8, and wherein amino acid residue 990 is D.

6. The genetically modified yeast of any one of claims 1 to 5, wherein said gene required for expressing a polypeptide having E2 ubiquitin-conjugating enzyme activity (EC:2.3.2.23) is:

a. an inactivated UBC13 gene encoding an amino acid sequence having at least 80% amino acid sequence identity to SEQ ID No.: 14, or

b. a mutant UBC13 gene encoding a mutant polypeptide, wherein the amino acid sequence of the polypeptide has 71 residues or less and has at least 80% amino acid sequence identity to SEQ ID No.: 16.

7. The genetically modified yeast of any one of claims 1 to 6, wherein the mutant gene encoding a glycerol kinase is a transgene; or said mutant gene is a genetically edited endogenous GUT1 gene.

8. The genetically modified yeast of any one of claims 3 to 6, wherein the inactivated KGD1, KGD2, LPD1 and UBC13 genes are genetically edited endogenous genes.

9. A method for enhancing glycerol metabolism in a yeast strain comprising:
   a. modifying an endogenous GUT1 gene to encode a mutant polypeptide having glycerol kinase activity (EC:2.7.1.30), wherein the amino acid sequence of said mutant polypeptide has at least 80% amino acid sequence identity to SEQ ID No.: 4, and wherein said sequence comprises an E572Q mutation; and
   b. inactivating one or more endogenous genes selected from among a KGD1, KGD2, and LPD1 gene in said yeast, or modifying an endogenous KGD1 gene in said yeast, wherein the modified gene encodes an amino acid sequence having at least 80% amino acid sequence identity to SEQ ID No.: 8, and wherein said amino acid sequence comprises an A990D mutation; and
   c. inactivating an endogenous UBC13 gene in said yeast, or modifying an endogenous UBC13 gene in said yeast, wherein the modified gene encodes an amino acid sequence of no more than 71 residues and having at least 80% amino acid sequence identity to amino acid residues 1-71 of SEQ ID No.: 14 or 16.

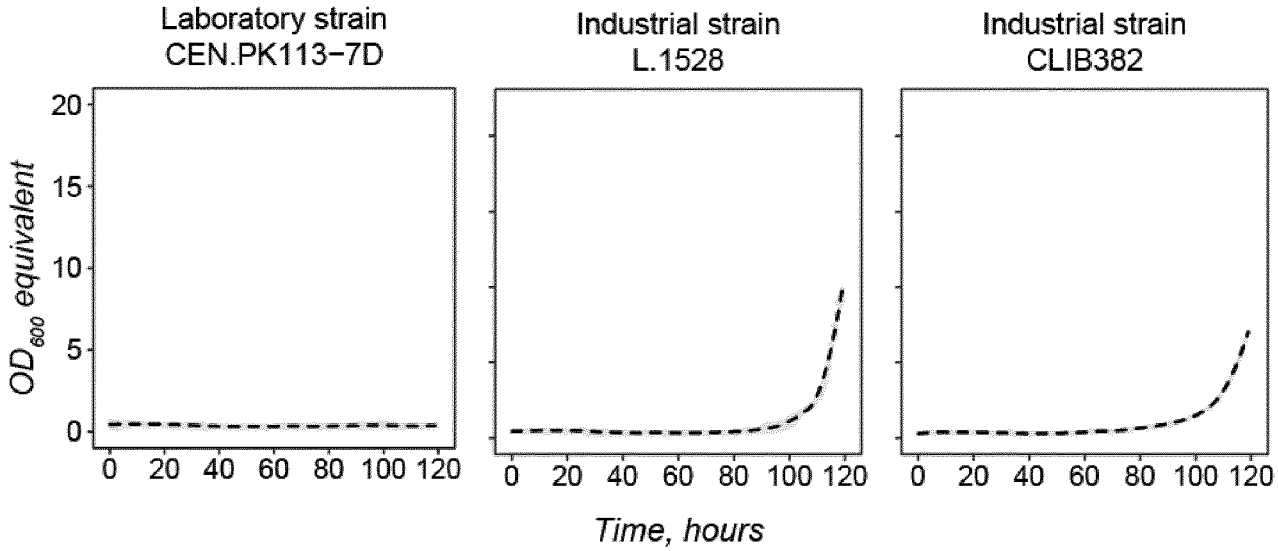
10. A method for culturing cells of the genetically modified yeast according to any one of claims 1 to 8 comprising the steps of:
   a. introducing the cells into a defined cultivation medium comprising a carbon source to produce a cell culture,
   b. cultivating the cell culture of (a) under aerobic growth conditions;
   wherein at least 80% by weight of the carbon source is glycerol.

11. A method for culturing cells according to claim 10, wherein the medium comprises 10 – 60 g/L glycerol.

12. Use of a genetically modified yeast with improved glycerol catabolism according to any one of claims 1 to 8, as a cell factory.

13. Use of a genetically modified yeast with improved glycerol catabolism according to claim 12, wherein said cell factory is provided with a culture medium comprising glycerol as sole carbon source.

Figure 1


Laboratory strain
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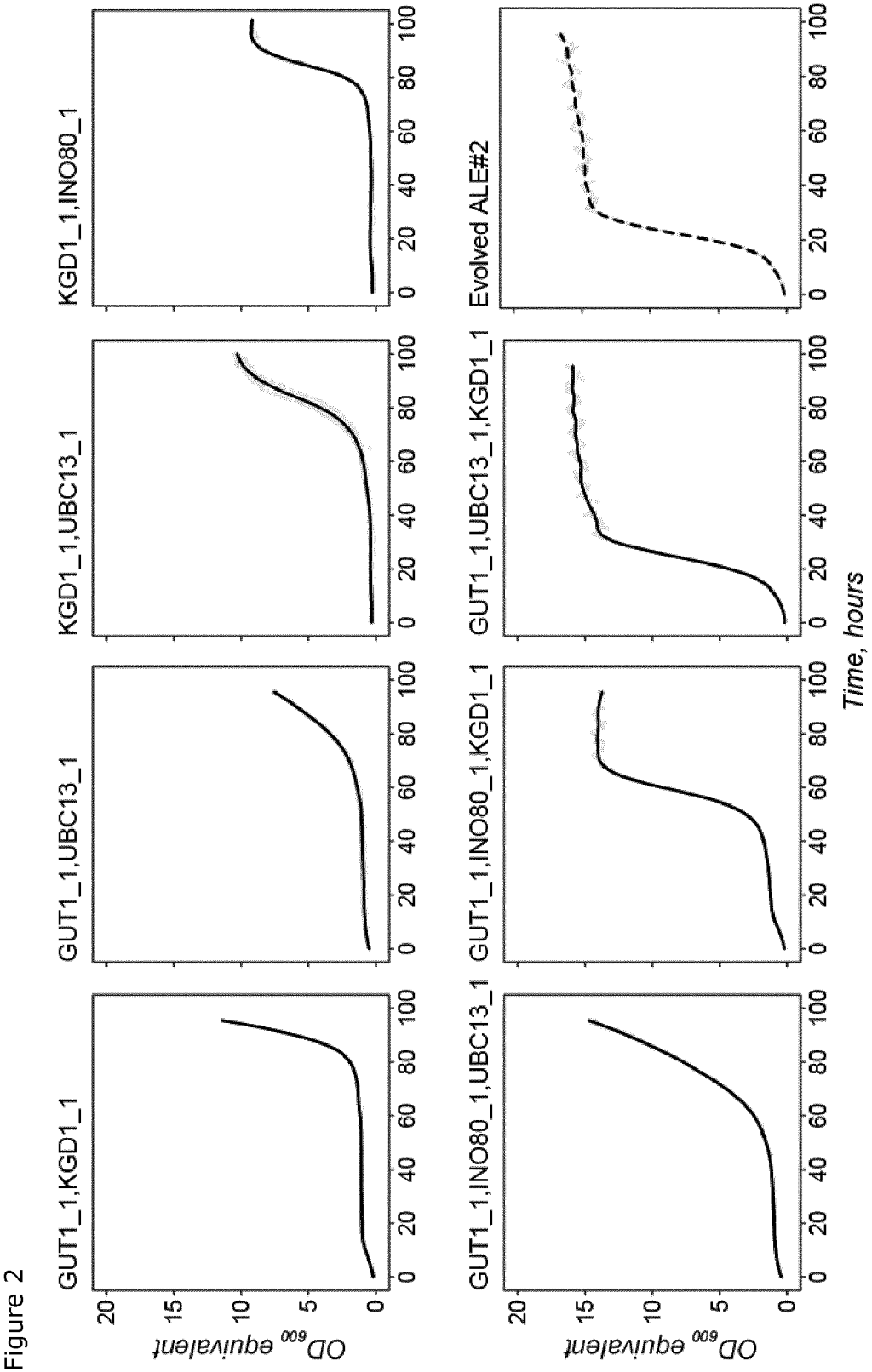
Industrial strain
L.1528
Industrial strain
CLIB382
OD600 equivalent
0
5
10
15
20
0 20 40 60 80 100 120
Time, hours

Figure 2


GUT1_1,KGD1_1
GUT1_1,UBC13_1
KGD1_1,UBC13_1
KGD1_1,INO80_1
GUT1_1,INO80_1,UBC13_1
GUT1_1,INO80_1,KGD1_1
GUT1_1,UBC13_1,KGD1_1
Evolved ALE#2
OD600 equivalent
Time, hours
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5
10
15
20
0
20
40
60
80
100

Figure 3

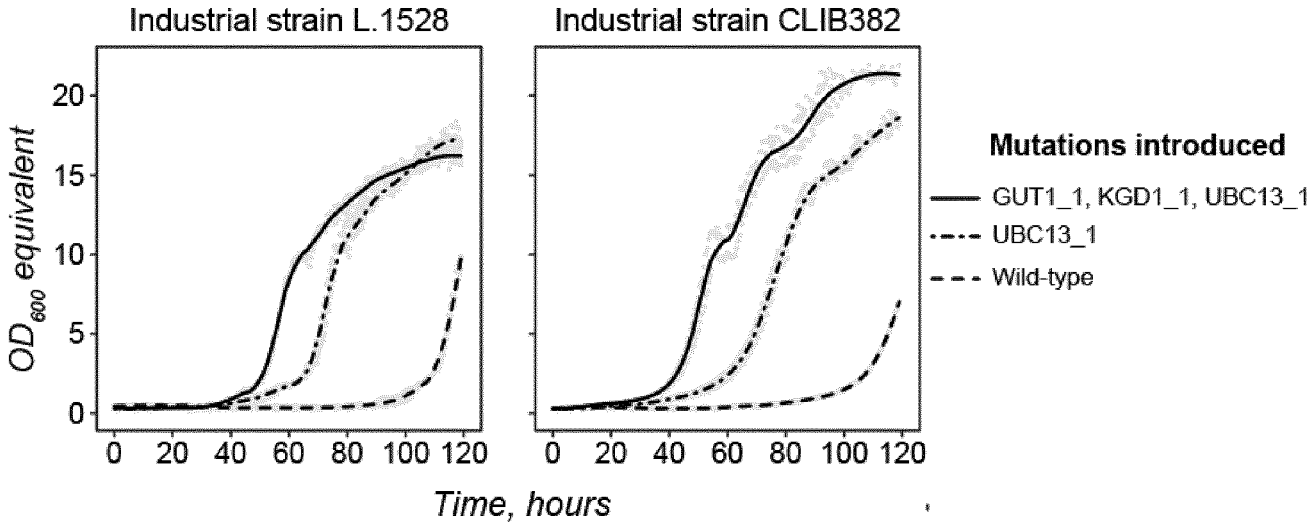



Figure 4

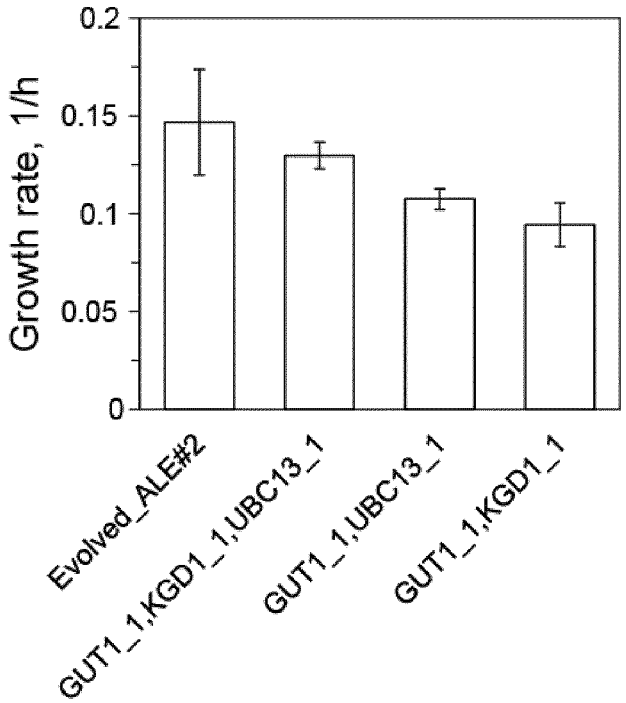


Figure 5

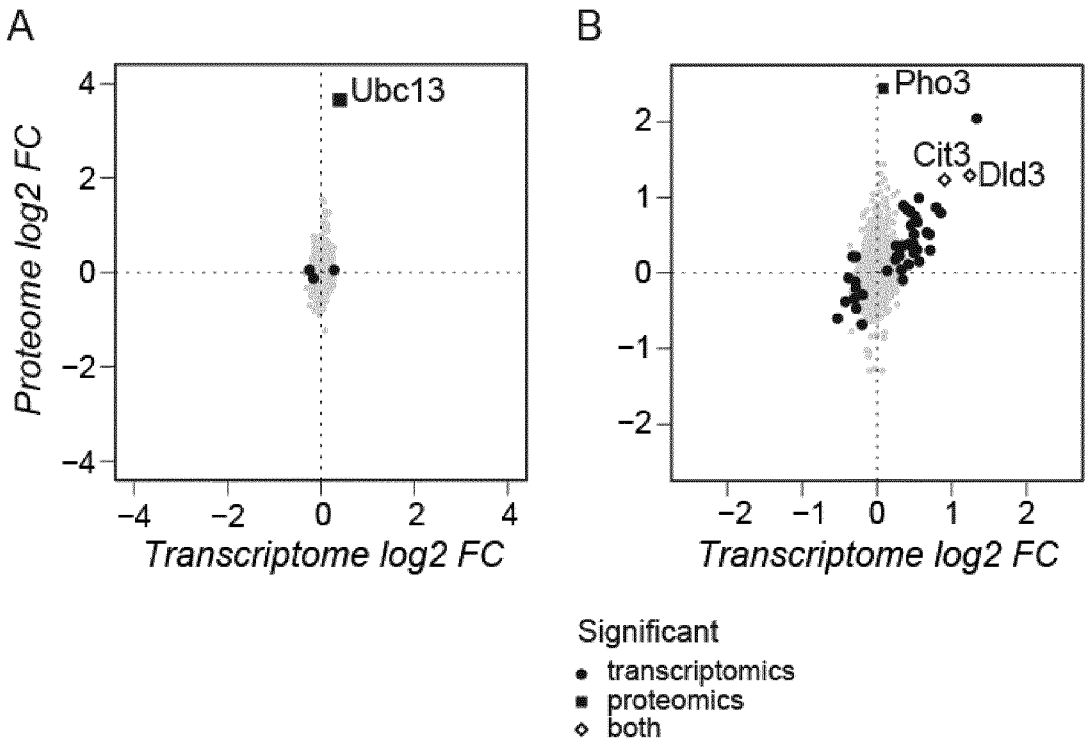
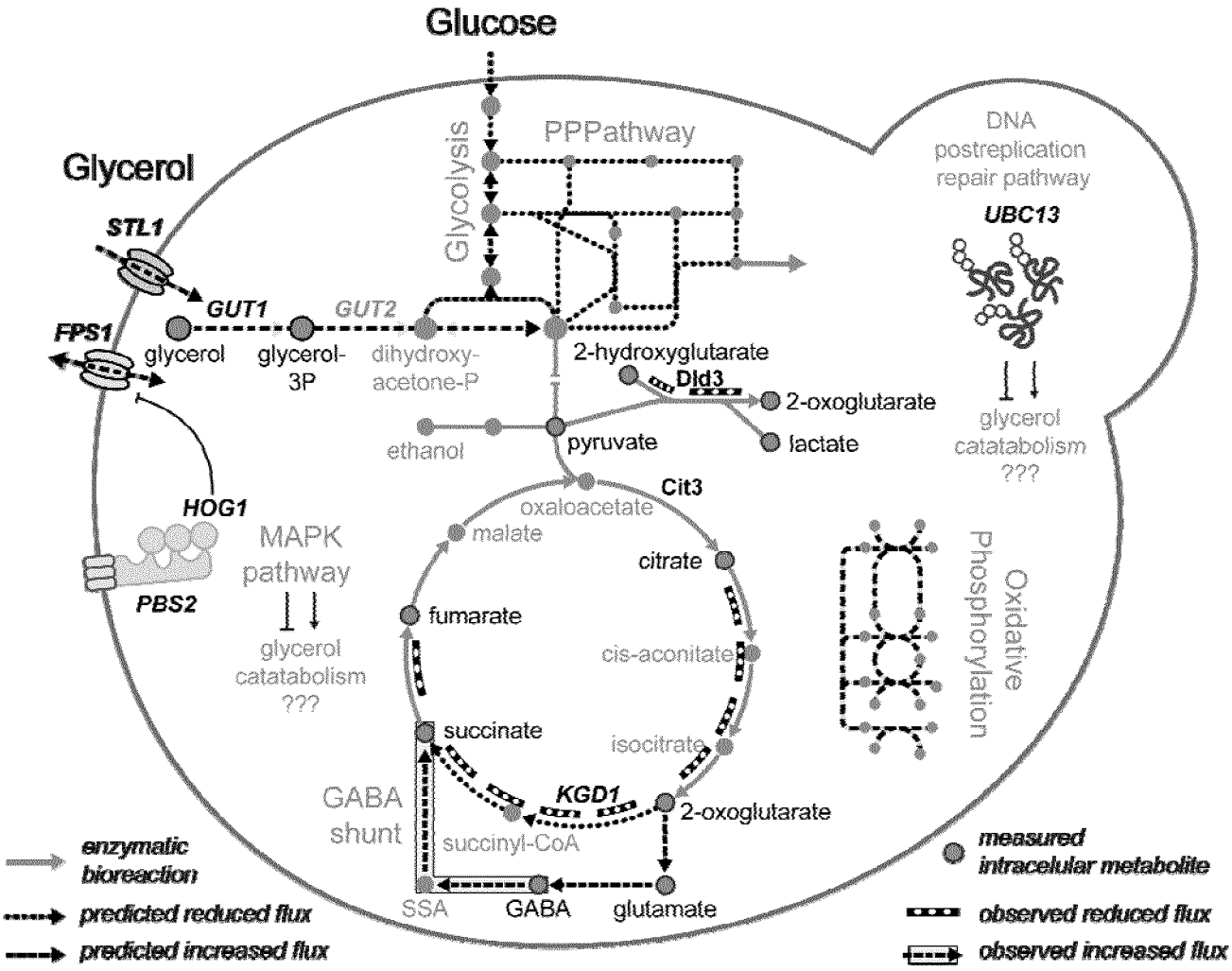

A
B
Ubc13
Pho3
Cit3
Dld3
Proteome log2 FC
Transcriptome log2 FC
Transcriptome log2 FC
Significant
transcriptomics
proteomics
both

Figure 6


Glucose
Glycerol
STL1
FPS1
GUT1
GUT2
glycerol
glycerol-3P
dihydroxy-acetone-P
Glycolysis
PPPathway
2-hydroxyglutarate
Dld3
2-oxoglutarate
lactate
ethanol
pyruvate
Cit3
oxaloacetate
malate
citrate
fumarate
cis-aconitate
succinate
isocitrate
KGD1
2-oxoglutarate
succinyl-CoA
GABA shunt
SSA
GABA
glutamate
HOG1
PBS2
MAPK pathway
glycerol catatabolism ???
DNA postreplication repair pathway
UBC13
glycerol catatabolism ???
Oxidative Phosphorylation
enzymatic bioreaction
predicted reduced flux
predicted increased flux
measured intracelular metabolite
observed reduced flux
observed increased flux

# INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2018/050291

A. CLASSIFICATION OF SUBJECT MATTER
INV. C12N1/16 C12N1/18 C12N1/19 C12N9/12 C12N9/02
C12N9/10 C12P1/02 C12P7/06
ADD.
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
C12N C12P

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
EPO-Internal, BIOSIS, Sequence Search, FSTA, WPI Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

| Category* | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
|---|---|---|
| A | JUNG J.-Y. ET AL: "Production of 1,2-Propanediol from Glycerol in Saccharomyces cerevisiae", JOURNAL OF MICROBIOLOGY AND BIOTECHNOLOGY, vol. 21, no. 8, 19 May 2011 (2011-05-19), pages 846-853, XP055037051, ISSN: 1017-7825, DOI: 10.4014/jmb.1103.03009 the whole document<br>----- | 1-13 |
| A | WO 2016/008819 A1 (UNIV JACOBS BREMEN GMBH [DE]) 21 January 2016 (2016-01-21) the whole document figure 1 claims 1,4,9<br>----- | 1-13 |

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[X] Further documents are listed in the continuation of Box C.

[X] See patent family annex.

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| Date of the actual completion of the international search | Date of mailing of the international search report |
|---|---|
| 13 March 2018 | 20/03/2018 |

| Name and mailing address of the ISA/ | Authorized officer |
|---|---|
| European Patent Office, P.B. 5818 Patentlaan 2<br>NL - 2280 HV Rijswijk<br>Tel. (+31-70) 340-2040,<br>Fax: (+31-70) 340-3016 | van de Kamp, Mart |

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C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

| Category* | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
|---|---|---|
| A | SWINNEN S. ET AL: "Re-evaluation of glycerol utilization in Saccharomyces cerevisiae: characterization of an isolate that grows on glycerol without supporting supplements", BIOTECHNOLOGY FOR BIOFUELS, vol. 6, no. 1, 8 November 2013 (2013-11-08), page 157, XP021168764, ISSN: 1754-6834, DOI: 10.1186/1754-6834-6-157 the whole document | 1-13 |
| A | SWINNEN S. ET AL: "Genetic determinants for enhanced glycerol growth ofSaccharomyces cerevisiae", METABOLIC ENGINEERING, vol. 36, 10 March 2016 (2016-03-10), pages 68-79, XP029593880, ISSN: 1096-7176, DOI: 10.1016/J.YMBEN.2016.03.003 cited in the application the whole document | 1-13 |
| A | KLEIN M. ET AL: "Towards the exploitation of glycerol's high reducing power inSaccharomyces cerevisiae-based bioprocesses", METABOLIC ENGINEERING, vol. 38, 14 October 2016 (2016-10-14), pages 464-472, XP029804998, ISSN: 1096-7176, DOI: 10.1016/J.YMBEN.2016.10.008 the whole document | 1-13 |
| A | OCHOA-ESTOPIER A. ET AL: "Kinetic analysis of a Saccharomyces cerevisiae strain adapted for improved growth on glycerol: Implications for the development of yeast bioprocesses on glycerol", BIORESOURCE TECHNOLOGY, vol. 102, no. 2, 13 August 2010 (2010-08-13), pages 1521-1527, XP027581475, ISSN: 0960-8524 the whole document | 1-13 |

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C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

| Category* | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
|---|---|---|
| T | KLEIN M. ET AL: "Glycerol metabolism and transport in yeast and fungi: established knowledge and ambiguities : Glycerol catabolism in yeast", ENVIRONMENTAL MICROBIOLOGY, vol. 19, no. 3, 30 January 2017 (2017-01-30), pages 878-893, XP055369533, GB ISSN: 1462-2912, DOI: 10.1111/1462-2920.13617 the whole document ----- | |

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# INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No
PCT/EP2018/050291

| Patent document cited in search report | Publication date | Patent family member(s) | Publication date |
|---|---|---|---|
| WO 2016008819 A1 | 21-01-2016 | DE 112015003261 A5 | 27-04-2017 |
| | | EP 3169768 A1 | 24-05-2017 |
| | | WO 2016008819 A1 | 21-01-2016 |